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Design, construction and function of a multicopy display vector using fusions to the major coat protein of bacteriophage M13

(Bovine pancreatic trypsin inhibitor-VIII protein fusion; single-stranded DNA phage; recombinant DNA)

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SUMMARY

Incorporation of numerous copies of a heterologous protein (bovine pancreatic trypsin inhibitor; BPTI) fused to the mature major coat protein (gene *VIII* product; VIII) of bacteriophage M13 has been demonstrated. Optimization of the promoter, signal peptide and host bacterial strain allowed for the construction of a working vector consisting of the M13 genome, into which was cloned a synthetic gene composed of a *lac* (or *tac*) promoter, and sequences encoding the bacterial alkaline phosphatase signal peptide, mature BPTI and the mature coat protein. Processing of the BPTI-VIII fusion protein and its incorporation into the bacteriophage were found to be maximal in a host bacterial strain containing a *prlA/secY* mutation. Functional protein is displayed on the surface of M13 phage, as judged by specific interactions with antiserum, anhydrotrypsin, and trypsin. Such display vectors can be used for epitope mapping, production of artificial vaccines and the screening of diverse libraries of proteins or peptides having affinity for a chosen ligand. The VIII display phage system has practical advantages over the III display phage system in that many more copies of the fusion protein can be displayed per phage particle and the presence of the VIII fusion protein has little or no effect on the infectivity of the resulting bacteriophage.

INTRODUCTION

Filamentous bacteriophage have been used to display protein fragments (Smith, 1985) and antigenic peptides (de la Cruz et al., 1988) as insertions into the *III*-encoded protein (III). Further, fusion to III has been used to display libraries of random peptides (Scott and Smith, 1990; Devlin et al., 1990; Cwirla et al., 1990), a single-chain antibody (McCafferty et al., 1990), human growth hormone (Bass et al., 1990) and part of the HIV *gag* product (Tsunetsugu-Yokota et al., 1991). Phage attachment to bacterial pili and subsequent infection require the function of

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Abbreviations: aa, amino acid(s); *afg-1*, gene encoding the VIII s.p.-BPTI-VIII fusion protein; *afg-2*, gene encoding the BAP s.p.-BPTI-VIII fusion protein; BAP, bacterial alkaline phosphatase; BPTI, bovine pancreatic trypsin inhibitor; BSA, bovine serum albumin; *Δ*, deletion; FAb, F(ab) fragment of an antibody; IPTG, isopropyl-β-D-thiogalactopyranoside; *lac*, promoter of the *E. coli* lactose operon; $lacO_s$, symmetric *lac* operator; nt, nucleotide(s); NRS, normal rabbit serum; oligo, oligodeoxyribonucleotide; OmpA, outer membrane protein A; PAGE, polyacrylamide-gel electrophoresis; PEG, polyethylene glycol; pfu, plaque-forming unit(s); *phoA*, gene encoding BAP; PMSF, phenylmethylsulfonyl-fluoride; *prlA/secY*, gene encoding the SecY (secretion) protein or PrlA (protein localization) protein; *rbs*, ribosome-binding site; SDS, sodium dodecyl sulfate; *sec*, genes encoding proteins of the secretory pathway; *tac*, a hybrid promoter containing nt sequences from the *E. coli trp* and *lac* promoters; TBS, Tris-buffered saline (10 mM Tris pH 7.4/150 mM NaCl); TE, Tris · EDTA (10 mM Tris pH 7.4/0.1 mM EDTA); *III*, gene that encodes the III coat protein of M13; *VIII*, gene encoding the VIII coat protein of M13.

III, which may be hampered in the above vectors, and which is present only at five copies per phage. An alternative display fusion protein in bacteriophage is the major coat protein (VIII) which is only 50 aa long and present at approx. 3000 copies per particle, being the predominant phage protein.

Display of peptides and proteins on the phage opens new possibilities for gene screening, epitope mapping, generation of immunogenic substrates, determination of antibody-peptide specificity, and the display of protein domains for further manipulation and analysis. Phage display vectors have the potential to enable the engineering of proteins so as to achieve a high affinity for almost any ligand. This undertaking involves phage display, mutagenesis of the parental protein gene, and selection of clones having desirable binding properties to the target.

The aim of this work was to display a functional and accessible heterologous protein fused to the major coat protein of bacteriophage. Independent of these studies, Kang et al. (1991) have described a system in which Fab molecules were fused to the phage major coat protein. A comparison of their display vector, which involves the use of helper phage, and the simpler system described here will be addressed.

RESULTS AND DISCUSSION

(a) Construction of major coat protein display vector

The starting synthetic gene, *afg-1* (*a*protinin *f*usion *g*ene), was assembled from 18 synthetic oligos (obtained from Genosys) after the methods of Kim et al. (1989). It comprises: a *lac* promoter, a symmetrized $lacO_s$ operator, an *rbs*, DNA encoding the VIII signal peptide-mature BPTI-mature VIII, translational terminators and a transcription terminator. Essentially the gene encodes a 'sandwich' of mature BPTI within the unprocessed VIII. Since the III signal peptide had been shown to be functional in the context of peptide- and protein-III fusions (see INTRODUCTION), it was considered feasible for the VIII signal peptide to function similarly in the context of the BPTI-VIII fusion.

Ligated segments of the *afg-1* gene were cloned into the unique *Hin*cII site of vector pGem-3Zf(−) (Promega, Madison, WI) to generate MB20 (Table I). In vitro transcription/translation of the genes contained within the pGem-3Zf(−) and MB20 vectors was performed with a prokaryotic system and [^{35}S]methionine (Amersham). PAGE of the labelled products and fluorography demonstrated two protein species (data not shown). β-Lactamase was expressed by each vector, while a 14.5-kDa species was observed only in MB20. This size is consistent with an unprocessed fusion protein resulting from expression of the *afg-1* gene.

TABLE I

VIII fusion phage vectors

Fusion phage[a]	Vector[b]	Promoter[c]	Signal peptide[d]	Fusion product[e]
MB1	M13	–	–	–
MB20	pGEM	*lac*	VIII	BPTI-VIII
MB26	pGEM	*tac*	VIII	BPTI-VIII
MB27	M13	*lac*	VIII	BPTI-VIII
MB28	M13	*tac*	VIII	BPTI-VIII
MB42	pGEM	*tac*	BAP	BPTI-VIII
MB48	M13	*lac*	BAP	BPTI-VIII
MB49	M13	*tac*	BAP	BPTI-VIII
MB56	M13.8ML	*lac*	BAP	BPTI-VIII
fk-SHO-BPTI	M13	*III*	III	BPTI-III

[a] Designations of the major coat fusion protein display phage. MB1 is a vector consisting of M13mp18 into which *Bam*HI and *Sal*I recognition sites have been introduced for the insertion of *afg* within the intergenic region. fk-SHO-BPTI is a BPTI-III display phage (B.L. R., submitted for publication).

[b] M13, M13mp18 (Norrander et al., 1983); pGem, pGem-3Zf(−) (Promega, Madison, WI). M13.8ML is a derivative of M13mp18 in which the Met start codon within the natural *VIII* has been mutated to a Leu codon.

[c] Promoter contained within *afg* (fusion protein gene). *lac*, promoter of the *E. coli* lactose operon; *tac*, a hybrid promoter containing nt sequences from the *lac* promoter and the *trp* promoter of *E. coli*; *III*, the promoter of M13 *III*.

[d] Signal peptide contained within the unprocessed s.p.-BPTI-VIII fusion. VIII signal peptide aa sequence: Met, Lys, Lys, Ser, Leu, Val, Leu, Lys, Ala, Ser, Val, Ala, Val, Ala, Thr, Leu, Val, Pro, Met, Leu, Ser, Phe, Ala. BAP signal peptide aa sequence (see Fig. 1). III signal peptide aa sequence: Met, Lys, Lys, Leu, Leu, Phe, Ala, Ile, Pro, Leu, Val, Val, Pro, Phe, Tyr, Ser, His, Ser.

[e] The fully processed fusion protein consisting of BPTI-VIII (see section c) or BPTI-III (B.L. R., submitted for publication).

Vector MB1 was used for synthesis of the *afg* product in the presence of the gene products of the M13 genome, allowing for the incorporation of the BPTI-VIII fusion protein into the coat during phage production. MB1 was constructed from M13mp18 (Norrander et al., 1983) by introducing *Bam*HI and *Sal*I sites (by site-directed mutagenesis) at nt 6001–6006 and 6428–6433, respectively. The *Bam*HI-*Sal*I fragment encompassing the *afg-1* synthetic gene, was recloned into the intergenic region of MB1 (replacing the *lacZ* gene and multiple cloning site) to generate MB27 which contains the entire M13 genome plus the fusion gene. The presence of identical nt sequences within a single vector is not desirable due to the possibility of homologous recombination and vector DNA rearrangements. The nt sequence homology between the natural *VIII* sequence and the synthetic *VIII* sequence contained within the fusion gene were significantly reduced by using alternative codons in the latter.

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(b) In vivo synthesis of the *afg-1* product

The pGem-based vectors were utilized in the analysis of fusion protein synthesis and processing since the M13-based vectors allow a potential avenue of escape for the fully processed product (i.e., incorporation into phage), while the pGem-based vector products are predicted to be retained in the cell whether processed or not. *E. coli* strain XL1 blue (Stratagene, La Jolla, CA) containing the pGem-based vector MB20 or MB26 (see Fig. 2) or infected with phage derived from the M13-based vector MB27 (data not shown), demonstrated the presence of a single 14.5-kDa protein species (when bacterial lysates were analyzed by Western-blot hybridization following SDS urea PAGE). This size is identical to that seen following in vitro transcription translation and led us to suspect that the fusion protein product was not being processed. No incorporation of fusion protein into the coat of bacteriophage derived from MB27 grown in XL1-blue was observed, probably a consequence of the processing defect.

Most bacterial proteins that are translocated through or into the plasma membrane travel via the *sec*-dependent pathway (reviewed by Wickner et al., 1991); however, the M13 procoat is translocated via a *sec*-independent pathway (reviewed by Wickner, 1988). The *sec*-independent secretion of the procoat protein requires elements contained in both the signal peptide and mature coat protein (Kuhn et al., 1987; Kuhn, 1988). It has been possible to insert 173 aa of OmpA between the signal peptide and mature coat protein resulting in a processed membrane-bound fusion protein. However, the *sec*-independent properties of the resulting fusion were lost (Kuhn, 1988). Our observations differ from this result in that insertion of BPTI into the M13 procoat resulted in a molecule which was not processed, at least in the strains we tested. Interestingly, the synthetic VIII signal peptide was shown to be functional when used to replace the signal peptide of β-lactamase (data not shown). It is likely that the VIII signal peptide does not function in the context of the BPTI-VIII fusion protein, suggesting that the BPTI moiety plays a role in this phenomenon.

(c) Optimizing fusion protein processing and expression

To alleviate the apparent processing problem demonstrated by MB20 or MB26 and MB27 the DNA encoding the VIII signal peptide was exchanged for a fragment (from *phoA*) encoding the signal peptide BAP of (Inouye et al., 1982). The choice of the BAP signal peptide reflects our hypothesis that a *sec*-dependent mechanism may be required to process and translocate an VIII-fusion protein and the fact that the BAP signal peptide has been shown previously to export BPTI when expressed in *E. coli* (Marks et al., 1986). The resulting constructs containing the *afg-2* gene (BAPs.p.-BPTI-VIII) (Fig. 1) were designated MB42 in the Gem-based vector and MB48 (*lac* promoter) or MB49 (*tac* promoter) in the M13-based vector.

Expression and partial processing of the BAP-BPTI-VIII fusion protein was observed in XL1-blue cells transfected with MB42 (Fig. 2) or infected with phage derived from MB48 and MB49 (data not shown). Lysates of the infected or transfected cells demonstrated two protein species by Western-blot analysis migrating at 14.5 kDa and 12 kDa (Fig. 2), which is consistent with processing activity.

The *lac* promoter sequence was replaced with a *tac* promoter sequence in an attempt to increase expression of the *afg* gene product, generating vectors MB26, MB28 and MB49 (Table I). Bacterial expression of the fusion protein was essentially equal with either promoter (data not shown).

(d) Effect of *prlA* mutation on fusion protein processing

The *E. coli prlA/secY* mutation has been shown to suppress export defects in bacterial protein secretion (Liss et al., 1985) and was considered a potential aid in the processing and incorporation of the BPTI-VIII fusion protein into bacteriophage. Strain SE6004 (*prlA*, F^-) was crossed with XL1-blue to generate the strain designated PECF01 {relevant genotype: *araD*139, Δ(*argF-lac*)U169, *rpsL*-150(Str^R), *relA* 1, *flbB*5301, *deoC*1, *ptsF*25, *rbsR*, *prlA*-4,[F′ *proAB*$^+$, *lacI*^{Q} ZΔM15, Tn*10*(*tet*^{R})]} containing both the *prlA* mutation and an F episome to enable phage infection. PECF01 demonstrated an increased ability to process the *afg-2*-encoded fusion protein. A greatly enhanced ratio of the processed (12-kDa) to unprocessed (14.5-kDa) protein (Fig. 2) and an enhanced incorporation of the fusion protein into bacteriophage was observed (section e) when the *prlA* host was compared to the parental strain (mc 4100) and XL1-blue. Replacement of the VIII signal peptide with that from BAP somewhat alleviated the processing problem but efficient processing was only observed in a strain containing a *prlA/secY* mutation. Strain PECF01 was utilized for the production of the fusion protein and its incorporation into the display phage.

(e) Incorporation of fusion protein into bacteriophage

In vivo synthesis of the fusion protein was demonstrated in PECF01 cells (data not shown) when infected with MB48 phage for 30 min followed by an addition of 0.5 mM IPTG, and a growth period of 2 h post-infection. Phage were purified by PEG precipitation, solubilization in TE buffer containing 0.1% Sarkosyl (Lin et al., 1980) followed by either reprecipitation with PEG or phage banding by centrifugation in CsCl, to give a pure phage preparation. The protease inhibitor PMSF was added to the buffers at a concentration of 1 mM.

Phage were analyzed by PAGE followed by silver staining or electrotransfer and Western-blot analysis with

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5'-GGATCC ATTCCCCATCCCC CTG TTGACA ATTAATCATCGGCTCG TATAAT
   BamHI|                   -35        tac              -10

GTGTGG AATTGTGAGCGCTCACAATT GAGCTCCATGGGAGAAAATAAA-
       lacO, operator       |SacI|        rbs

| M | K | Q | S | T | I | A | L | A | L | L | P | L | L -
|-21|-20|-19|-18|-17|-16|-15|-14|-13|-12|-11|-10| -9| -8-
|ATG|AAA|CAA|AGC|ACG|ATC|GCA|CTG|GCA|CTC|TTA|CCG|TTA|CTG-
                    | PvuI |
<--------------- BAP signal peptide -------------------
| F | T | P | V | T | K | A | R | P | D | F | C | L | E -
| -7| -6| -5| -4| -3| -2| -1| 1 | 2 | 3 | 4 | 5 | 6 | 7 -
|TTT|ACC|CCT|GTG|ACA|AAA|GCC|CGT|CCG|GAT|TTC|TGT|CTC|GAG-
                                |AccIII|        | XhoI |
------------------------------><----- BPTI ----------------
| P | P | Y | T | G | P | C | K | A | R | I | I | R | Y -
| 8 | 9 | 10| 11| 12| 13| 14| 15| 16| 17| 18| 19| 20| 21-
|CCA|CCA|TAC|ACT|GGG|CCC|TGC|AAA|GCG|CGC|ATC|ATC|CGC|TAT-
                  | ApaI |        |BssHII |
-------------------------------------------------------
| F | Y | N | A | K | A | G | L | C | Q | T | F | V | Y -
| 22| 23| 24| 25| 26| 27| 28| 29| 30| 31| 32| 33| 34| 35-
|TTC|TAC|AAT|GCT|AAA|GCA|GGC|CTG|TGC|CAG|ACC|TTT|GTA|TAC-
                        | StuI |                | AccI |
-------------------------------------------------------
| G | G | C | R | A | K | R | N | N | F | K | S | A | E -
| 36| 37| 38| 39| 40| 41| 42| 43| 44| 45| 46| 47| 48| 49-
|GGT|GGT|TGC|CGT|GCT|AAG|CGT|AAC|AAC|TTT|AAA|TCG|GCC|GAA-
                  | EspI |                    | EagI |
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| D | C | M | R | T | C | G | G | A | A | E | G | D | D -
| 50| 51| 52| 53| 54| 55| 56| 57| 58| 59| 60| 61| 62| 63-
|GAT|TGC|ATG|CGT|ACC|TGC|GGT|GGC|GCC|GCT|GAA|GGT|GAT|GAT-
      | SphI |                | NarI |
--- BPTI ------------------------------><----- Mature VIII -
| P | A | K | A | A | F | N | S | L | Q | A | S | A | T -
| 64| 65| 66| 67| 68| 69| 70| 71| 72| 73| 74| 75| 76| 77-
|CCG|GCC|AAG|GCG|GCC|TTC|AAT|TCT|CTG|CAA|GCT|TCT|GCT|ACC-
   |     SfiI      |                  | HindIII|
-------------------------------------------------------
| E | Y | I | G | Y | A | W | A | M | V | V | V | I | V -
| 78| 79| 80| 81| 82| 83| 84| 85| 86| 87| 88| 89| 90| 91-
|GAG|TAT|ATT|GGT|TAC|GCG|TGG|GCC|ATG|GTG|GTG|GTT|ATC|GTT-
                              |     BstXI     |
-------------------------------------------------------
| G | A | T | I | G | I | K | L | F | K | K | F | T | S -
| 92| 93| 94| 95| 96| 97| 98| 99|100|101|102|103|104|105-
|GGT|GCT|ACC|ATC|GGG|ATC|AAA|CTG|TTC|AAG|AAG|TTT|ACT|TCG-
                                                  |AsuII|
---------------------------------------------- Mature VIII -
| K | A | S | . | . | . |
|106|107|108|   |   |   |
|AAG|GCG|TCT|TAA|TGA|TAG|GGTTACC AGTCTA AGCCCGC CTAATGA
                        |BstEII|
--------------> Stop codons            Transcription

GCGGGCT TTTTTTTT ATCGA    GACCTGCA GGTCGAC-3'
  Terminator                       |SalI|
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Fig. 1. Nucleotide sequence of the BAP s.p.-BPTI-VIII phage display fusion gene (*afg-2*) and aa sequence of the protein. Unique restriction sites and salient points within the gene and protein are indicated. Cleavage of the nascent fusion protein by signal peptidase-I is predicted to occur between Ala^{-1} and Arg^{+1} (A/R). *tac* is the hybrid *trp-lac* promoter.

anti-BPTI rabbit serum. A single immunoreactive species of approx. 12 kDa was present in the induced MB48 phage but not in control phage (Fig. 3a). Silver staining of the same phage preparations (Fig. 3b) readily visualized the major coat protein in both phage preparations and an extra species of approx. 12 kDa was detected only in the induced MB48 phage. Analysis of a serially diluted MB48 phage preparation (data not shown) was used to estimate the ratio of these two proteins, which was typically in the range of 1:50 to 1:100 (fusion protein : coat protein). Since phage contain approx. 3000 copies of the major coat protein, induced MB48 phage contain at least tens of copies of the BPTI-VIII fusion per phage particle. A time course for fusion protein incorporation during phage production showed it to be maximal at 2 h (data not shown).

The incorporation of BPTI-VIII fusion protein into phage is likely to be controlled by the competition between the natural VIII and the introduced *afg*-encoded product. To shift this equilibrium so as to favor the incorporation of the fusion protein, the natural *VIII* was mutated. The Met start codon (ATG) within the natural *VIII* of MB48 was converted, by site-directed mutagenesis, to a Leu codon (CTG) giving phage variant MB56. Translational initiation of the natural *VIII*-encoded mRNA still occurred, but at a diminished rate, as judged by an approx. tenfold reduction in total phage production by MB56. The relative incorporation of the *afg* product was significantly increased in this phage variant, demonstrating the highest incorporation of the fusion-VIII protein seen (Fig. 3,a and b). PAGE analysis (Fig. 3c) of the MB56-derived phage showed that the fusion protein was present at a ratio of approx. 1:30 relative to the coat protein, an average of 100 copies per phage particle. We assume that the MB56 variant contains approx. 3000 copies of the major coat protein, since the amount of encapsidated DNA remains unaltered and there are no changes in the mature coat protein.

(f) Accessibility and functionality of the displayed protein

To determine whether the BPTI domain was displayed and accessible in the VIII-display phage we determined the effect on phage titer of adding anti-BPTI IgG. The addition of anti-BPTI IgG to the control phage (M13mp18) caused no loss in titer, while a significant drop was observed with the BPTI-III-display phage (fK-SHO-BPTI; B. L. Roberts submitted for publication) with or without the addition of protein A agarose beads (Table II). Such a drop in titer from addition of antibody alone is expected since III is involved in bacterial infection. Phage MB48 demonstrated a significant decrease in titer only when both the specific antibody and protein A agarose beads were added. The addition of NRS or protein A agarose had no effect on phage titer for any of the VIII-display phage species (data not shown). MB56 phage and certain batches of MB48

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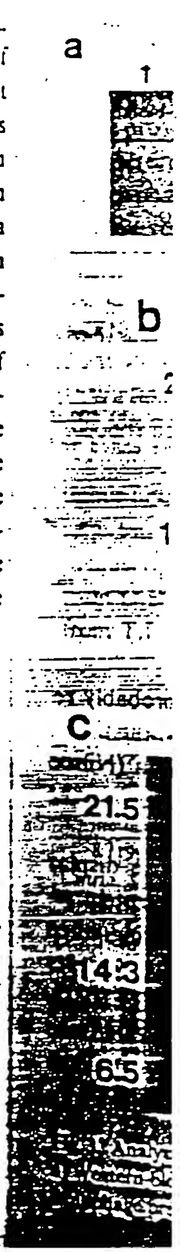

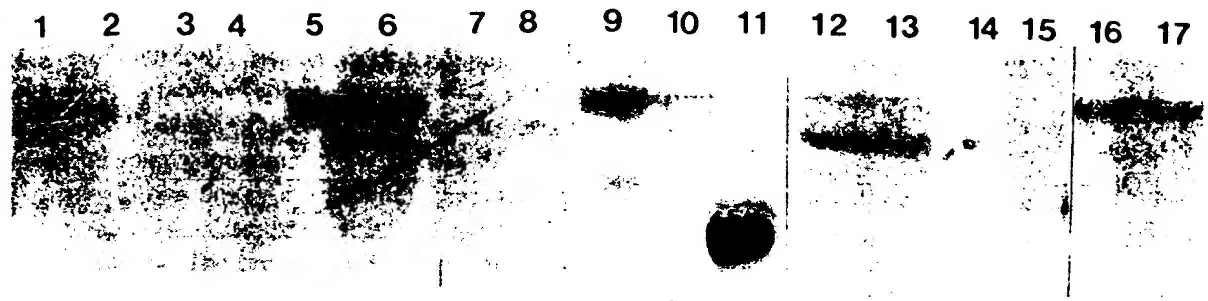

Fig. 2. Analysis of fusion protein processing in different *E. coli* strains. This figure demonstrates that efficient processing of the BAP s.p.-BPTI-VIII fusion protein occurs most readily in a bacterial strain containing a *prlA/secY* mutation. Western-blot analysis of cell lysate proteins separated using 0.1% SDS/6 M urea/15% PAGE, electrotransferred to Immobilon and probed with anti-BPTI rabbit serum followed by horse radish peroxidase-conjugated sheep anti-rabbit IgG and peroxidase substrate. Lanes: **1** and **2**, MB26 (VIII s.p.-BPTI-VIII) in strain MC4100; **3** and **4**, strain MC4100 control; **5** and **6**, MB42 (BAP s.p.-BPTI-VIII) in strain XL1-blue; **7** and **8**, strain XL1-blue control; **9** and **10**, MB26 in strain XL1-blue; **11**, BPTI marker (0.1 μg); **12** and **13**, MB42 in strain PECF01 (*prlA*); **14** and **15**, PECF01 (*prlA*) control; **16** and **17**, MB26 in strain PECF01 (*prlA*); **2**, **4**, **6**, **8**, **10**, **12**, **14** and **17**, expression without IPTG; **1**, **3**, **5**, **7**, **9**, **13**, **15** and **16** with 0.5 mM IPTG.

phage (batch 4 in Table II for example) with higher levels of BPTI-fusion protein incorporation demonstrated a precipitation reaction on overnight incubation with anti-BPTI IgG but not with NRS (data not shown). Presumably this is due to multivalent interactions with the antibodies.

To determine the functionality of the major coat protein-displayed BPTI molecule, binding assays were performed using either anhydrotrypsin agarose beads or trypsin agarose beads (inactive and active forms of a natural target of BPTI). The amount of phage bound to a given bead was determined by elution with a low pH buffer followed by neutralization and the quantitation of phage in the eluate as plaque-forming units (Table IIIa). BPTI display phage bound to anhydrotrypsin and trypsin agarose beads (Table IIIb) 50-fold and 250-fold, respectively, greater than the control (M13mp18) phage. The relative binding of the BPTI-VIII display phage was greater with trypsin beads due, in part, to a reduction in the nonspecific binding of the

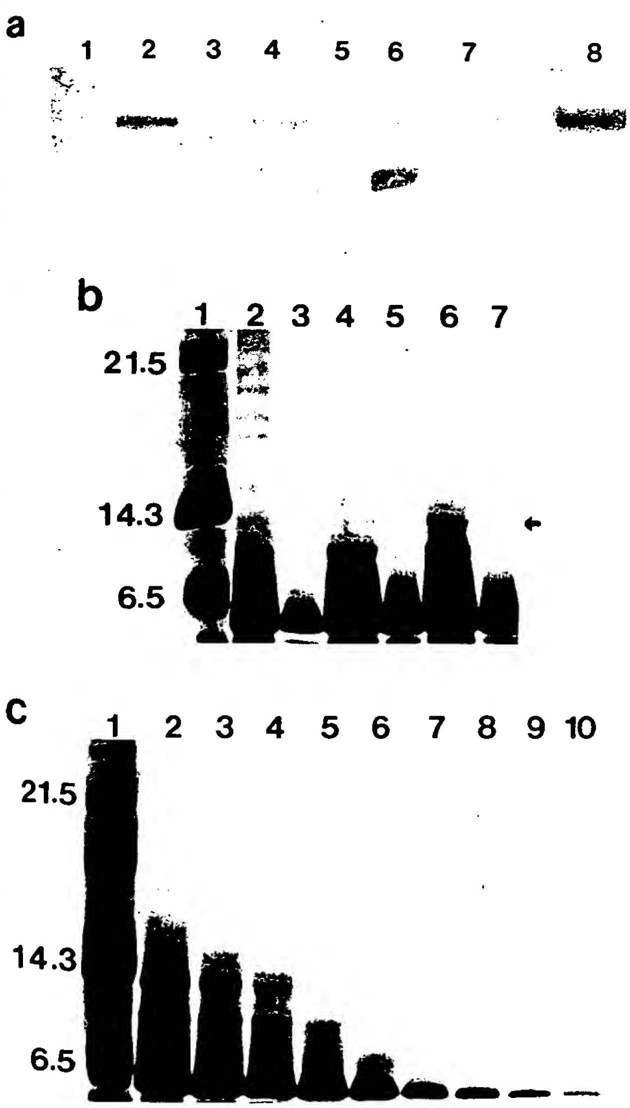

Fig. 3. Analysis of BPTI-VIII display phage by PAGE. (Panel **a**) Western-blot analysis of display-VIII phage. Lanes: **1**, M13mp18 (control) phage prepared using TE Sarkosyl, plus PMSF and CsCl gradient-centrifugation; **2**, MB48 (BPTI-display) phage prepared as in lane **1**; **3**, as in lane **2** with one fifth the loading; **4**, as lane **2** but prepared in the absence of PMFS; **5**, as lane **4** with one fifth the loading; **6**, BPTI marker; **7**, as lane **2**; **8**, MB56 (high incorporation) phage prepared as in lane **1**. For Western-blot analysis 10^{11} pfu's were loaded per lane and electrophoresed through a 15% polyacrylamide gel containing 0.1% SDS and 6 M urea. The proteins were electrotransferred to Immobilon (Millipore) and incubated with primary antibody (anti-BPTI rabbit serum) and secondary antibody (horse radish peroxidase-conjugated sheep anti-rabbit IgG) to visualize the BPTI domain of the fusion protein. (Panel **b**) Silver-stained polyacrylamide gel of display phage strains. Lanes: **1**, markers; **2** and **3**, M13mp18 (non-display) phage; **4** and **5**, MB48 (BPTI-display) phage; **6** and **7**, MB56 (high-incorporation BPTI-display) phage; **2**, **4** and **6**, phage loading of 5×10^{10}; **3**, **5** and **7**, phage loading of 1×10^{10}. Arrow indicates position of the fusion protein. (Panel **c**) Silver-stained 15% polyacrylamide gel with twofold serially diluted MB56 phage. Lanes: **1**, markers; **2**, undiluted (5×10^{10} pfu); **3**, twofold dilution; **4**, fourfold; **5**, eightfold; **6**, 16-fold; **7**, 32-fold; **8**, 64-fold; **9**, 128-fold; **10**, 256-fold.

TABLE II

Effect of anti-BPTI IgG on BPTI-VIII display phage[a]

Phage strain[b]	Residual titer[c] (% of input)		Eluted titer[d] (% of input)
	+ anti-BPTI	+ anti-BPTI + protein A	Eluted phage
M13mp18	98	92	7×10^{-4}
fk-SHO-BPTI	26	21	6.0
MB48[e]	90	36	0.8
MB48[f]	60	40	2.6

[a] Methods. Display phage and controls were diluted to 1.5×10^9 pfu per µl in TBS containing 1 mg per ml BSA. To 100 µl of the diluted phage was added 3 µg of purified anti-BPTI rabbit IgG followed by an incubation at room temperature for 2 to 4 h. To 5 µl of pre-washed protein A-agarose beads (binding capacity 25 mg/ml) were added the phage-antibody mixture and incubation was continued overnight in an end-over-end rotator. The beads were washed five times with 500 µl of TBS containing 0.1% Tween. Finally the bound phage were eluted from the beads with 500 µl of 0.1 M glycine (pH 2.2) (Parmley and Smith, 1988) followed by neutralization with Tris buffer. Samples were taken from each stage of the procedure and the phage titer determined. Control experiments with protein A alone, NRS and protein A plus NRS were also performed. Experiments were performed at least twice. Phage titers were determined in duplicate over a range of dilutions.

[b] See Table I.

[c] Total pfu remaining in the supernatant following addition of either anti-BPTI IgG or anti-BPTI IgG plus protein A-agarose beads, expressed as % of the starting number of phage.

[d] Total pfu acid eluted from the anti-BPTI IgG: protein A-agarose-bead complex, expressed as % of the starting number of phage.

[e] MB48 batch 3.

[f] MB48 batch 4.

TABLE III

Binding of display phage[a]

(a) Binding to anhydrotrypsin

Experiment 1

Strain[b]	Eluted phage[c]	Relative binding[d]
M13mp18	0.2	1.0
fK-SHO-BPTI	7.9	40.0
MB48	11.2	56.0

Experiment 2

Strain	Eluted phage	Relative binding
M13mp18	0.3	1.0
fK-SHO-BPTI	12.0	40.0
MB56	17.0	57.0

(b) Binding to trypsin

Strain	Eluted phage	Relative binding
M13mp18	5×10^{-4}	1.0
fK-SHO-BPTI	1.0	2000.0
MB48	0.13	260.0

[a] Methods. Display phage and controls were diluted to a concentration of 1.5×10^9 pfu/µl in TBS containing 1 mg/ml of BSA. To 30 µl of the diluted phage was added 5 µl of anhydrotrypsin-agarose beads (Pierce, binding capacity 60 nmol/ml gel) or trypsin-agarose beads (Pierce, 14 U/ml gel) and allowed to incubate at room temperature end-over-end for 2–4 h. Beads were briefly pelleted then washed five times with 500 µl of TBS containing 0.1% Tween. The bound phage were eluted and quantitated as in the legend to Table II.

[b] See Table I.

[c] Total pfu acid eluted from protease-agarose beads, expressed as % of the starting number of phage.

[d] Protease binding of the BPTI display phage relative to that of the nondisplay phage, M13mp18.

phage since the absolute level of binding was actually lower. The BPTI-display phage failed to bind to human neutrophil elastase, another serine protease (data not shown), demonstrating specificity for trypsin in the binding assays.

(g) Conclusions

(*1*) A heterologous protein domain has been demonstrated to be displayed when fused to the major coat protein of M13 bacteriophage. The BPTI-VIII fusion protein has been shown to be incorporated into the phage coat and to be displayed and functional as evidenced by the ability to bind to an enzymatically active target. The multicopy display of proteins or peptides fused to VIII of filamentous bacteriophage will be useful in epitope mapping, production of artificial vaccines, and the screening of synthetically diverse libraries for a protein having affinity for a chosen ligand.

(*2*) The inability of the *afg-1* fusion product to be processed probably resulted from a disfunction of the VIII signal peptide (Laforet et al., 1989). The observable, but still poor processing of the *afg-2* fusion product probably resulted from an insufficient rate of translocation relative to the rapid folding of the BPTI moiety in bacteria (Nilson et al., 1991). This was alleviated to a large extent in the *prlA/secY* mutant strain.

(*3*) A comparison of III-based bacteriophage display vectors and that based upon the major coat protein reported here, raises some points worth considering when screening phage display libraries for proteins or peptides with novel or improved binding properties. First, the BPTI-VIII fusion protein has been shown to be incorporated into phage, typically at 30 to 60 copies per particle, which when fractionating a variegated display population of phage with a gradient of eluent (e.g., decreasing pH), may allow for a sharper transition to be achieved between bound and unbound display phage than with the III display phage

which has at the most five copies. Secondly, the fusion of a peptide or protein to III frequently results in a reduction of infectivity for the bacteriophage (Smith, 1985; Parmley and Smith, 1988). During the course of screening a large heterologous display population, there is a risk that phage with less desirable binding properties but higher infectivity will outgrow those with more desirable binding properties but lower infectivity. The separation of display and infectivity is achieved in the VIII display phage.

(4) Kang et al. (1991) have recently demonstrated the ability to display an FAb fragment fused to the major coat protein of M13. The leader sequence was the *pelB*-encoded peptide and incorporation into phage relied upon the use of a helper phage. Electron microscopy demonstrated the display of between 1 and 24 FAb fusion proteins per phage. The system described here demonstrates the wider applicability of the major coat protein as a display framework, enables a greater incorporation of fusion proteins into the coat of the bacteriophage, and is a simpler system overall. A negative feature of a helper-phage-based display system is the prospect of generating phage which display molecules of interest separated from their encoding gene, i.e., the helper phage genome has been encapsidated instead of the display vector.

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